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## **Postprandial changes of amino acid and acylcarnitine concentrations in dried blood samples**

Fingerhut, R ; De Jesus Silva Arevalo, G ; Baumgartner, M R ; Häberle, J ; Rohrbach, M ; Figueroa, A W ; Fresse, E M ; Polanco, O L ; Torresani, T

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# Postprandial changes of amino acid and acylcarnitine concentrations in dried blood samples

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**Abstract** Blood sampling for newborn screening cannot be standardized as for example blood collection in adults after an overnight fast. Therefore the influence of postprandial changes and individual variation is valuable information for the assessment of sensitivity and specificity of newborn screening for certain disorders. We have analyzed 92 pairs of dried blood samples taken pre- and one hour postprandially, respectively. We have determined the mean increase in metabolite concentration and calculated its significance. Individual variation after an overnight fast in healthy adults ( $n=3$ ) was between 12 and 32% (SD). Postprandial increases of acylcarnitines were mostly not significant and not exceeding 10%. Postprandial increase of amino acids was highly significant for most proteinogenic amino acids, but not for all. With the collected data we were able to estimate that mainly decreased levels of methionine and, to a lesser extent, of free carnitine could be “masked” by

postprandial increases of the respective metabolites, and could therefore lead to false negative results for the detection of disorders of cobalamin metabolism and carnitine transporter deficiency.

## Abbreviations

Newborn screening	NBS
Inborn errors of metabolism	IEM
Dried blood samples	DBS
Thyroid stimulating hormone	TSH
17 $\alpha$ -hydroxyprogesterone	17-OHP
Medium-chain triglycerides	MCT
Standard deviation	SD

## Introduction

Newborn screening (NBS) for inborn errors of metabolism (IEM) and endocrinopathies is one of the most effective tools of preventive health care. NBS is normally regionally organized and ideally it should cover every newborn of the respective population of that region. Special screening tests in selected risk populations might be justified. However, due to the ever-increasing worldwide mobility and interracial marriages, these justifications will vanish over time. All NBS programs normally use native capillary or venous whole blood dried on special filter paper [the so-called dried blood spots, or dried blood samples (DBS)] taken by heel prick or venous puncture, respectively. The age at sampling might differ from program to program, e.g., between 36 and 72 h in Germany and the 6th day of life in England, and there are some differing recommendations concerning early sampling for babies discharged early, as well as the time of second sampling in preterms. These differences, such as age at blood collection, birth weight, or

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gestational age, could be overcome by age, birth weight, and gestational-age-related reference values. However, only few publications on these data exist, and they often take only the variation of a single variable into account (Cavedon et al 2005, Meyburg et al 2001, Chace et al 2003, Honzik et al. 2005, Corte and Venta 2010, Duran 2008, Matern 2008). The influence of some medications, such as dopamine and steroids upon thyroid-stimulating hormone (TSH) (Wilber and Utiger 1969, Brabant et al. 1989, Haugen 2009) and  $17\alpha$ -hydroxyprogesterone ( $17$ -OHP), respectively, is well known (Rohrer et al. 2003). For children who need a blood transfusion, all screening programs recommend that a first sample should be taken before transfusion and a second sample 3–14 days after the last transfusion. Nutrition is another factor that can influence metabolite concentrations, in particular, substitution with carnitine, total parenteral nutrition, high caloric glucose infusion (Shih 1984), and other food additives such as medium-chain triglycerides (MCT), can influence the concentrations of free carnitine, acylcarnitines, and amino acids and might thereby cause false positive and false negative screening results. In the normal clinical chemistry setting, reference ranges for basal metabolite concentrations are normally related to blood collections after an overnight fast. Obviously, this strategy is impossible for newborns and NBS. Therefore, NBS programs give no recommendations on the time of blood collection concerning the last feed. Due to international ethical guidelines, it is impossible to conduct studies in newborns and children to elucidate these open questions. For this study, we had the opportunity to reevaluate pairs of samples (DBS) from newborns that were taken pre- and 1-h postprandially to prove or disprove a portovenous shunt (DD: galactosemia) (Nishimura et al. 2004, Sodhi et al. 2007). With the data presented here, we can estimate the variation of amino acids and acylcarnitines due to the fed/fasted state. This will also give a rough estimate of the bias that is included in all reference ranges for amino acids and acylcarnitines in NBS programs.

## Materials and methods

Blood was taken by heel prick or venous puncture and spotted onto filter paper before and 1 h after feeding, either in the course of follow-up tests after a positive newborn screen for galactosemia in order to exclude or confirm a portovenous shunt ( $n=80$ ), or from healthy volunteers ( $n=12$ ; age 19–48 years). Newborns and volunteers were healthy, on a normal diet and had no medications. None of the newborns in the study had galactosemia or any kind of liver disease. To determine individual variation of amino acids and acylcarnitines in the fasted state, capillary blood from three healthy volunteers was taken after an overnight

fast on 11, 13, and 15 consecutive days, respectively. Amino acids and acylcarnitines were measured after extraction with methanol, without derivatization, either with the Neogram kit from Perkin Elmer, on an API2000 tandem mass spectrometer from SCIEX; or with the Neobase kit from Perkin Elmer, on a TQD tandem mass spectrometer from Waters. Measured acylcarnitines and amino acids are listed in Table 1. For statistical analysis,

**Table 1** List of acylcarnitines and amino acids measured from dried blood spots (DBS)

acylcarnitines	amino acids
free carnitine (C0)	phenylalanine
acetylcarnitine (C2)	tyrosine
propionylcarnitine (C3)	methionine
butyrylcarnitine (C4)	valine
isovalerylcarnitine (C5)	leucine/isoleucine/ alloisoleucine/ hydroxyproline*
hexanoylcarnitine (C6)	citrulline
octanoylcarnitine (C8)	ornithine
decanoylcarnitine (C10)	arginine
dodecanoylcarnitine (C12)	alanine
tetradecanoylcarnitine (C14)	glycine
palmitoylcarnitine (C16)	proline
stearoylcarnitine (C18)	
pentenoylcarnitine (C5:1)	
octenoylcarnitine (C8:1)	
decenoylcarnitine (C10:1)	
decadienoylcarnitine (C10:2)	
dodecenoylcarnitine (C12:1)	
tetradecenoylcarnitine (C14:1)	
tetradecadienoylcarnitine (C14:2)	
hexadecenoylcarnitine (C16:1)	
octadecenoylcarnitine (C18:1)	
octadecadienoylcarnitine (C18:2)	
malonylcarnitine (C3DC)/ hydroxybutyrylcarnitine (C4-OH) *	
methylmalonylcarnitine (C4DC)/ hydroxyisovalerylcarnitine (C5-OH) *	
glutaryl carnitine (C5DC)/ hydroxyhexanoylcarnitine (C6-OH) *	
methylglutaryl carnitine (C6DC)	
hydroxytetradecanoylcarnitine (C14-OH)	
hydroxyhexadecanoylcarnitine (C16-OH)	
hydroxyhexadecenoylcarnitine (C16:1-OH)	
hydroxyoctadecanoylcarnitine (C18-OH)	
hydroxyoctadecenoylcarnitine (C18:1-OH)	

\*Isobaric compounds that cannot be distinguished by this method

SPSS 16.0 was used. The *t* test for paired samples was used to calculate the significance of pre/postprandial differences of amino acid and acylcarnitine concentrations.

## Results

The individual variation for free carnitine and acylcarnitines is negligible [standard deviation (SD) range for free carnitine 3.1–4.3  $\mu\text{mol/L}$ ; for acetylcarnitine 1.0–2.5  $\mu\text{mol/L}$ ; for all other acylcarnitines  $<0.3 \mu\text{mol/L}$ ]. The individual variations for amino acids in the fasting state are all between 12% and 32% (Table 2). The postprandial increase was highly significant for most proteinogenic amino acids; for acylcarnitines, with some exceptions, there was no or no significant increase (see Table 3).

To estimate the influence of postprandial increases on the recall rate for certain disorders, we calculated the centile for the respective metabolites from 84,665 initial NBS samples; day of sampling between 3 and 5 days. Since the 99.9th centile is a widely used cutoff in NBS programs, we took the concentrations of the respective metabolites at the 99.9th centile, added and accordingly subtracted the mean postprandial increase (pp-incr.) of the respective metabolite, and determined the centiles for these concentrations (see Fig. 1 for schematic description). These calculations give the “cut-off  $-\times\%$  pp-incr.” and “cut-off  $+\times\%$  pp-incr.” and the respective number of samples [ $(n-)$  and  $(n+)$ ] within these ranges for each metabolite, respectively. Since we have no information on the fed/fasted state of the newborns, we assume that there is an equal distribution of samples taken from directly after feeding to samples taken shortly before feeding. We then simplified this model by assuming 50% of the samples  $(n-)$  and  $(n+)$  were taken preprandially and 50% postprandially, respectively. With this simplification, we calculated the number of samples from the  $n-$  group that would give a recall, if all samples would have been taken postprandially [ $\Rightarrow (r+)$ ], and the number of

samples from the  $n+$  group that would not give a recall, if all samples would have been taken preprandially [ $\Rightarrow (r-)$ ]. The difference of these two numbers ( $r + \text{minus } r-$ ) in relation to the total number of recalls result in a factor (see Table 3) that gives a rough estimate of the influence of postprandial changes on the recall rate. A factor  $>1$  would argue in favor of a postprandial influence, a factor  $<1$  would argue against it. For those analytes, where decreased concentrations have pathological relevance (methionine, free carnitine, octadecanoylcarnitine), we made the same calculation also for the 0.1st centile.

## Discussion

Since blood collection for NBS cannot be standardized like blood collections in adults (normally drawn after an overnight fast), and because intensive studies in children are ethically not justifiable, all reference ranges generated for NBS comprise a certain bias. Our results derived by a model calculation can help to estimate the bias of amino acid and acylcarnitine determination to a certain extent. Amino acid and acylcarnitine concentrations may vary due to many different conditions: age, birth weight (Fingerhut et al. 2001), fed/fasted state, pre/postprandial time frame, individual variation, total parenteral nutrition, carnitine substitution. Some of these conditions can be controlled, such as age and birth weight, by age- and birth-weight-dependent reference ranges; others can be checked and dealt with by ordering a second sample after a certain time period (total parenteral nutrition, carnitine substitution). Individual variation is inherently included in reference ranges; however, the range of variation in a single individual is often unknown. Finally, fed/fasted state or pre/postprandial blood collection is uncontrollable in NBS.

The presented data show that mainly proteinogenic amino acids have a highly significant postprandial increase, which is, of course, not surprising; however, also ornithine, a nonproteinogenic amino acid, shows significant increase. From the acylcarnitines, only free carnitine, propionylcarnitine, and acylcarnitines associated to fat tissue (C18, C18:1) showed modest but slightly significant increases. The increase of propionylcarnitine might be explained by a combination of dietary uptake and systemic increase due to the increase in free carnitine. Increase of free carnitine alone cannot be the explanation, since these two metabolites are not consistently concurrently increased in the 84 sample pairs (data not shown).

However, for NBS, the increase in itself is not what is important, but the influence on the recall rate, false positive, and false negative results. To determine this influence, we attempted to estimate the impact on recall rates as described in the “Results” section and shown in Table 3 and Fig. 1.

**Table 2** Individual variation of amino acids in the fasted state (range;  $n=3$ )

compound	SD (%)
phenylalanine (p)	13.2–20.6
tyrosine (p)	17.8–20.2
methionine (p)	19.0–24.9
valine (p)	16.7–18.5
leucine (p)	17.4–20.8
citrulline (n)	12.3–23.8
ornithine (n)	22.4–31.6
arginine (p)	18.5–22.6
alanine (p)	17.1–25.8
glycine (p)	16.6–23.9
proline (p)	13.6–19.7

SD standard deviation, (p) proteinogenic amino acid, (n) non-proteinogenic amino acid

**Table 3** Mean postprandial increase of amino acids and acylcarnitines

compound	postprandial increase (%)	<i>P</i> value	estimated influence on the recall rate (factor)
phenylalanine (p)***	18	< 0.001	0.72
tyrosine (p)***	14	< 0.001	0.18
methionine (p)***	26	< 0.001	4.29 (increased) 1.84 (decreased)
valine (p)***	18	< 0.001	1.62
leucine <sup>a</sup> (p)***	25	< 0.001	5.19
proline (p)***	11	< 0.001	0.46
arginine (p)***	17	< 0.001	0.79
alanine (p)***	11	< 0.001	0.44
ornithine (n)**	13	0.007	0.45
glycine (p)*	9	0.062	0.08
free carnitine (C0)*	6	0.016	0.09 (increased) 0.09 (decreased)
propionylcarnitine (C3)*	7	0.039	0.01
octadecanoylcarnitine (C18)*	7	0.048	0.25 (increased) 0.00 (decreased)
octadecenoylcarnitin (C18:1)*	4	0.019	0.10
citrulline (n) (n.s.)	-	0.715	-
hexadecanoylcarnitine (C16) (n.s.)	-	0.077	-
other acylcarnitines (n.s.)	-	0.1–0.8	-

(p) proteinogenic amino acid, (n) nonproteinogenic amino acid, n.s. not significant ( $p > 0.05$ )

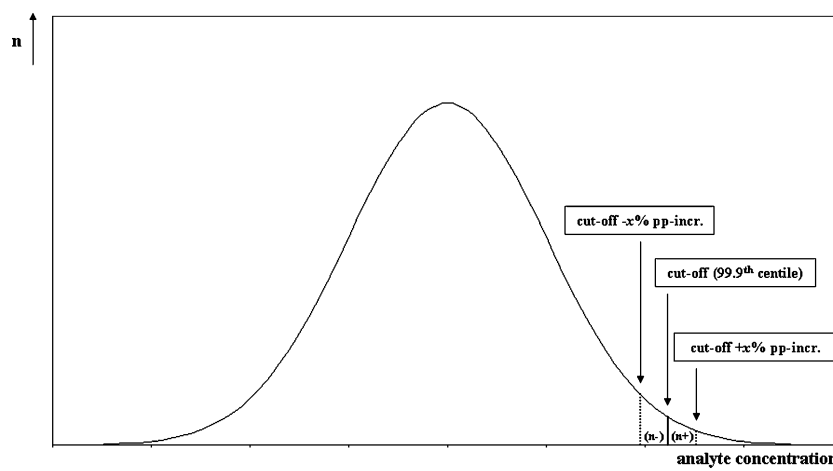
\*Significant ( $p \leq 0.05$ ); \*\*very significant ( $p \leq 0.01$ ); \*\*\*highly significant ( $p \leq 0.001$ )

<sup>a</sup> Sum of isobaric amino acids (leucine + isoleucine + alloisoleucine + hydroxyproline)

<sup>b</sup> Sum of isobaric acylcarnitines (glutaryl carnitine + hydroxyhexanoylcarnitine)

Fatty acid oxidation disorders and organic acidurias, which are detected through elevated concentrations of specific acylcarnitines, should be mainly unaffected. Only screening for propionic academia/methylmalonic acidemia might bear a borderline risk of increased recall and false positive rates. On the panel for NBS, so far, only two disorders are listed

that are detected through decreased levels of free carnitine or specific acylcarnitines. Carnitine transporter (OCTN2) deficiency with decreased free carnitine, and carnitine palmitoyl transferase I deficiency (CPT-I) with decreased levels of long-chain acylcarnitines (C16, C18). The detection rate of OCTN2 deficiency is known to be not



**Fig. 1** Model for estimating the influence of postprandial metabolite changes: *pp-incr.* postprandial increase, *cut-off -x% pp-incr.* centile for the concentration of the respective metabolite at the 99.9th centile minus the percentage of respective postprandial increase, *cut-off +x% pp-incr.* centile for the concentration of the respective metabolite at the 99.9th centile plus the percentage of respective postprandial increase, (*n-*)

number of samples within the concentration range for the respective between the 99.9th centile and the centile corresponding to cut-off -x% pp-incr., (*n+*) number of samples within the concentration range for the respective between the 99.9th centile and the centile corresponding to cut-off +x% pp-incr

100% sensitive, which can be due to postprandial increase of free carnitine; however, a more probable explanation might be the different body stores of free carnitine, which are dependent on the maternal levels of free carnitine during pregnancy and lactation. The sensitivity for CPT-I deficiency should not be influenced, since the most specific metabolite ratio  $[C0/(C16 + C18)]$  (Fingerhut et al. 2001) is more or less unaffected, because both denominator as well as numerator will increase postprandially.

For amino acidemias and urea-cycle disorders, the situation is more complex due to the greater number of amino acids that are increased postprandially. Disorders that might be affected are hyperphenylalaninemia (PKU/HPA), homocystinuria, disorders of cobalamin metabolism, maple syrup urine disease (MSUD), arginase deficiency, and hyperornithinemia. Since homocystinuria, with methionine as the marker metabolite, has an extremely low sensitivity when NBS samples are taken during the first 4 days of life, this can be neglected. The influence on PKU/HPA, MSUD, arginase deficiency, and hyperornithinemia can also be neglected, since postprandial increases are not specific, and the interpretation of the whole amino acid profile with slight increases of several amino acids can avoid unnecessary recalls. Only disorders of cobalamin metabolism, which are detected by decreased levels of methionine, are likely to give false negative results due to a possible postprandial increase of methionine into the low normal range. This is especially probable since methionine shows the highest postprandial increase of all metabolites tested.

In conclusion, postprandial increases of amino acids and acylcarnitines are partially significant and might be the cause for some false positive screening results. However, the danger for false negative results can only be suggested for disorders of cobalamin metabolism and, to a lesser extent, for OCTN2 deficiency, where decreased levels of methionine and free carnitine, respectively, are the only marker metabolites.

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